Reference: *Biol. Bull.,* **155**: 220-234. (August, 1978)

TENTACLE NUMBER IN CULTURED *HYDRA VIRIDIS*

STANLEY SHOSTAK, DANIEL MEDIC, JR., FREDERICK A. SPROULL AND CHARLES C. JONES

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

The term, *morphogen,* coined by Turing (1952), has recently become popular, and research on morphogens, fashionable. Morphogens are substances involved in morphogenesis (without growth), illustrated by evocators, thought to be active in induction. The recent popularity of the term is derived from its generality, which makes it suitable for formal mathematical treatment and for experimentation. Conceptual simplicity allows investigators simultaneously to pursue the morphogenic consequences of morphogens in computers and their real counterparts in simple experimental situations.

Hydra's linear cylindrical form, marked polarity, with tentacles at one end, a foot at the other, and a budding region near the middle, and its ability to regenerate as well as reproduce vegetatively by budding has attracted the attention of investigators interested in morphogens. The flourishing research on morphogens in *Hydra,* led by Schaller (1973, 1976a,b), has centered on the number of tentacles (tentacle number) produced in pieces of regenerating animals and buds.

Tentacle number is a convenient variable not suffering from the vagueness surrounding criteria for differentiation of hypostome or foot. Independent of diameter or length, a tentacle is identifiable as such. Questions concerning the number of tentacles present on an animal are readily separated from questions concerning their dimensions, and the data on number lend themselves to statistical treatment.

The history of experimental studies on tentacle number has been continuous for more than a decade, and has suggested that tentacle number is a complex variable influenced by many factors. Lesh-Laurie (1974) used tentacle number in her investigations of morphogenic roles of nucleic acids, and Yasugi (1974) demonstrated that the lithium ion promotes the regeneration of entire heads of hydras with reversed polarity, but with an actual reduction in tentacle number. Earlier, Lenicque (1967a, b; Lenicque and Lunblad, 1966) examined the ability of extracts of hydras and inhibitors of neurosecretory substances to alter tentacle numbers during regeneration; and while Lentz and Barrnett (1963) and Lesh and Burnett (1964) did not report counts of tentacle numbers, they clearly indicated an increase in tentacle number in some of the hydras treated with extracts of hydras thought to contain neurosecretory material.

Other factors may also influence tentacle number. Burnett (1961, 1967) has long argued for a morphogenic role of growth in *Hydra* and has experimented with agents that inhibit mitosis, among other activities. Webster (1967) asserts that Colcemid increases tentacle numbers in regenerating animals, but Corff and Burnett (1969) show that colchicine inhibits tentacle regeneration. Shostak and Tammariello (1969) show that the increased tentacle numbers found in Colcemid-treated animals can be due to the retention of buds that would otherwise have detached from parental hydras. Müller and Spindler (1971) attribute the dramatic increase in tentacle number found in animals treated with some of their extracts of hydras to the fusion of potentially separate heads, and Shostak (1977a) shows that fused head-ends can regenerate single heads with abnormally high tentacle numbers.

Tentacle number in normal animals has not been extensively studied. The most frequently cited report on the topic is that of Parke (1900) who concluded that (p. 702) "the number of tentacles possessed by a given Hydra varies in accordance with its age, size and doubtless other factors," and that "the number of tentacles possessed by a bud at the time that it is constricted off, is not usually the same as the number of tentacles that will be possessed by the same individual at the later period." Thus, the tentacle number would seem to be highly variable in individuals and extremely vulnerable to local conditions in the wild.

Hydras can be altered in several ways: via changes in temperature, feeding schedule, and wounding or grafting. Reducing the ambient temperature of a culture tends to increase the size of the average animal (Stiven, 1965; Park and Ortmeyer, 1972; Bisbee, 1973), and the frequency of feeding is well known for its proportional effect on growth (*e.g.*, Shostak, Bisbee, Ashkin and Tammariello, 1968). Wounding and grafting readily alter the size of animals with consequences for regeneration and budding (Shostak, 1972, 1977b).

In the present study, animals cultured under ordinary conditions for our laboratory, first supplied a base line pool of information on normal tentacle numbers and variation in the population. The change in this tentacle number on parental animals and on their own buds was then examined. Feeding schedules, temperature, and wounding and grafting were employed to alter hydras and the effects of these variable on tentacle number in both parents and buds was monitored.

Materials and Methods

Specimens of *Hydra viridis* of the large European variety, originally supplied by A. L. Burnett, were cultured in large Pyrex-brand baking dishes at room temperature, 21 to 26° C, fed daily on one to two day old brine shrimp nauplii, *Artemia* sp. (specimens supplied by pet dealer with uncertain species designation), and subjected to a complete change of medium about one hour after feeding (Loomis and Lenhoff, 1956). Experiments and observations were performed on individual animals kept in 55 mm Petri dishes in about 12 ml of culture medium (Shostak, Patel and Burnett, 1965). In experiments involving particular temperatures, animals were kept in incubators and culture medium was brought to the desired temperature before exchanging for old medium. Grafting was performed with the usual methods: human hair was used to skewer the freshly cut pieces together until healing between them was complete (Shostak, 1977b). Statistical methods were based on Snedecor (1956) and Sokal and Rohlf (1969), modified and combined in order to accommodate features of the present experiments. Where no other method is specified, the Student's *t*-test was employed to evaluate differences in paired comparisons.

Results

Tentacle numbers on parental hydras

The first objective was to determine the degree of variation in tentacle number found among hydras selected at random from the mass cultures. The sample of

TABLE I

Average tentacle number over eight days.

Month Batch	October		November	
	1	2	1	2
	7.9	7.0	7.0	7.0
	8.0	7.0	7.2	6.0
	7.1	7.0	6.0	6.0
	7.0	7.0	7.0	7.0
	7.1	7.0	7.8	6.0
	7.0	7.8	6.0	5.9
	8.0	6.1	6.1	7.0
	8.0	7.0	6.9	6.0
	7.0	6.0	6.0	7.0
	8.0	7.0	7.0	6.0
	6.0	7.0	6.0	7.0
Average for batches	7.38	6.90	6.64	6.44
Average for month	7.14		6.54	
Overall average	6.84			

hydras for study was obtained by taking "standard" animals (having two buds; Shostak, 1968) at random from the cultures and placing them individually in Petri dishes. The animals were maintained for 3 days at which time they were removed, while the buds which had detached that day, were retained. In October and November, 1976, 22 animals in two groups (batches) of 11 were collected. Every animal was fed *Artemia* nauplii in abundance and had its tentacles counted. Different observers scored the animals in the 2 months.

The range of tentacle numbers was 6 to 8 for the sample. The monthly and batch means did not differ significantly, as tested by the F-statistic. In terms of the components of variance, however, months accounted for nearly 36% of the variance, while batches within months accounted for 9%. The remaining variance, that within batches, accounted for more than half the variance. This degree of variation among animals amply justifies statements in the literature that tentacle number in *Hydra* varies under constant conditions and from month to month, and cautions one against attaching importance to small differences.

The next objective was to determine how much variation was due to daily changes in tentacle number on these same animals. Tentacle numbers were scored daily for 8 days, a period thought to be of suitable duration, inasmuch as cellular turnover on tentacles is complete in about 4 days, according to Campbell (1967). The average tentacle numbers for each of the 44 animals observed over the course of 8 days are listed in Table I. Of the 44 animals, 34 showed no change whatsoever, seven showed a change of one, one showed a change of two tentacles, and two animals showed two changes of one tentacle. Most of these changes were transient, however, being reversed on the day following the recorded change. Overall, four

TABLE II

Analysis of variance (two-way).

Source of Variation	Degrees of freedom	Sum of squares	Mean squares	F	Probability
Animals	43	149.2			
months	1	31.4	31.4	5.40	$0.25 > P > 0.10$
batches within months	2	11.6	5.80	2.18	$0.25 > P > 0.10$
animals within batches	40	106.2	2.66	66.50	$0.001 \gg P$
Days	7	0.4	0.06	1.50	$0.25 > P > 0.10$
Error	301	12.4	0.04		

animals lost one tentacle over the 8 days, and one gained a tentacle. The analysis of variance of these data (a two way analysis with the two levels within animals) is summarized in Table II. Variation between months and between batches within months in this table are comparable to the similar statistics calculated for the data accumulated on the first day alone as is the F-statistic. These similarities indicate how little change occurs over the period of eight days. Indeed, the mean squares for days is not significant and represents a very small component of variation. The mean square between animals within the same batch is the only term found to be significant, as one might have anticipated, given the large component of variation due to individuals in the first day's data.

The results emphasize the difficulty one has in attributing significance to small changes in tentacle number between randomly selected animals. The small, for the most part, reversible, and not significant changes in tentacle numbers over the course of a week's study are as reasonably attributed to human error in counting as to actual change. In practice, these experiments, which were done "blind", that is, without prior knowledge of the tentacle number on an animal at the time of counting, may have yielded larger changes in tentacle number than actual, but changes that are, nevertheless, not significant.

During the first week after a bud detaches, it grows and commences budding under the present conditions. The animals nearly double their surface area and begin budding generally by the second day (Shostak, 1968). By the end of the week, most of the animals would be considered "standard", possessing two developing buds. The average animal has produced about four detached buds by this time. Neither growth nor budding, therefore, along with time as such, have altered the tentacle numbers of animals significantly when cultured continuously under these conditions. Tentacle number is a stable characteristic of individual hydras.

Tentacle numbers on buds

The buds produced by the population of 44 parent animals were also scored for tentacle numbers while attached to these parents and on the day of detaching. The range of tentacle numbers on detached buds was 4 to 8, and the mean tentacle number for 403 buds collected was 6.26. Including the 44 parents, which were originally freshly detached buds themselves, 447 buds were collected. Their mean

TABLE III

Frequencies of tentacle numbers.

Tentacle number	Observed frequencies	Expected frequencies for normal distribution
3	0	0.089
4	3	4.96
5	65	64.77
6	184	197.80
7	174	150.64
8	21	27.54
9	0	1.20
10	0	0

tentacle number was 6.32 with standard deviation 0.79 tentacles. The observed frequencies and those expected from a normally distributed population are given in Table III.

The observed frequencies were tested for skewness and kurtosis using the sample statistics, g_1 and g_2, with their respective standard deviations (Sokal and Rohlf, 1969). Small negative values of both g_1 and g_2 were not significant $(0.4 > P > 0.2)$ as shown by the *t*-test with infinite degrees of freedom. The observed frequencies, therefore, are normally distributed, symmetrical and have no significant flattening or peaking compared to a normally distributed population with the same mean and standard deviation.

These conclusions are fairly apparent upon casual inspection of the frequencies in Table III. The observed data appear symmetrical around the values calculated for a normally distributed population. Of course, the "central limit theorem" permits the use of "the normal distribution to make statistical inferences about means of populations in which the items are not at all normally distributed," (Sokal and Rohlf, 1969, p. 130), especially when large sample sizes are employed. The importance of this demonstration of normality is that common parametric statistical tests, such as the F-test for which normal distributions of the sample data are assumed, can now be employed without bias to test inferences about different tentacle numbers between vegetatively produced populations of hydras.

The next objective was to determine if the tentacle number on buds varies as a function of the tentacle number on parents. The correlation coefficient, r, for the 403 buds on the 44 parents studied was found to be significant with a value of −0.14, indicating a loose general relationship between the tentacle number on buds and their parents, which, under the conditions employed, is negative. Actually, two of the three buds with only 4 tentacles were produced by parents having 8 tentacles, the remaining bud with 4 tentacles having been produced by a parent with 6 tentacles. Within the range of tentacle numbers (6–8) found on the present sample of parents, therefore, buds are evidently produced with tentacle numbers varying within a similar range (4–8) and broadly negatively correlated with the parent's tentacle number.

These data also made it possible to determine if tentacle numbers on buds vary with days of the week or the sequence with which buds are produced. Ten buds

TABLE IV

Average tentacle number on developing buds.

Month	Number of animals	Days of bud development on parents detached		
		1	2	3
Oct.	89	2.19	5.92	6.02
Nov.	89	3.54	6.29	6.38
Overall	178	2.86	6.11	6.20

produced on each of 5 weekdays were selected at random from the October and from the November samples. The mean tentacle number for each day did not change significantly as tested by the F-statistic ($0.25 > P > 0.10$) nor did the differences between the means for the two months. To test the possibility that the tentacle number of buds varies as a function of their sequence of formation, 14 parental animals in October and in November, which had complete records for up to the eighth bud, were chosen for the sample. Like days of the week, tentacle number did not change significantly with sequence as tested by the F-statistic. Even the first bud produced by the parents failed to have significantly more or less tentacles than subsequent buds.

The number of tentacles present on developing buds was recorded on each of the parent animals sampled in October and November, providing a record of the progress of each bud. The records of 93 buds in October and 89 in November spanned 3 days (2 days of development on the parent plus the day of detaching) and were used for the samples. (The sample for October was reduced to 89 by randomly eliminating four records in order to have samples of equal size.) Buds that were apparent, but had not yet developed tentacles, were recorded as having a tentacle number of zero. Presumably, when no record of a bud was made 2 days before its detachment, it had not begun developing or had evaded the observer's scrutiny due to small size. This difference would account for buds with records spanning 2 days and those spanning 3 days. No further difference is apparent in the tentacle numbers for these animals. The results are summarized in Table IV.

On inspection, these averages indicate that the greatest period of increase in tentacle number is before the second day of development, after which time tentacle number changes little. The analysis of variance of these data bears out this impression. Significant differences are found between days of bud development. Upon partitioning the variance between Day 1 *vs.* Day 2 and 3, and among Days 2 and 3 (each with one degree of freedom) one discovers that almost all the variance resides between Day 1 and the other days, Days 2 and 3 having only a fraction of the variation between them and a mean square which is not significant. This result pushes back the period during which animals may be said to have stable tentacle numbers to the day before detachment, at least under constant conditions.

Feeding schedule: effect on tentacle number of parents and buds

The frequency of feeding has well known effects on the growth and size of hydras and on the number of buds produced (Shostak *et al.*, 1968). The object of this experiment was to determine if animals fed with different frequencies underwent changes in their tentacle numbers or produced buds with different numbers of tentacles. In addition to the daily feeding schedule, animals were fed on two 3-days a week schedules (intended to approximately halve the caloric input of animals fed daily) and a twice-a-day schedule (intended to double the daily caloric intake). The two 3-days a week schedules were employed to provide animals which were cycling between periods of budding and nonbudding, and animals which were budding at a slow and continuous or diminishing rate. The experiment was carried out for 2 weeks in order to allow an adequate period for changes in tentacle number to develop.

Sets of 12 animals were fed Monday through Wednesday (MTW) and Monday, Wednesday and Friday (MWF) and the tentacles on the parents and on buds counted daily. The results (Table V) for parents and for buds of animals on the two different schedules did not differ significantly. Thus, the average tentacle numbers shown represent the pooled data for parents (column 2) and for buds (column 3). The average tentacle numbers for the parental animals, like the parents fed daily, show little change, a conclusion validated by the analysis of variance. The mean square for days was not significant ($0.75 > P > 0.50$), nor was linear regression ($P > 0.75$). The mean squares for animals within feeding schedules are, however, highly significant, as expected for animals taken at random from stock cultures.

Unlike the parents, the tentacle number on freshly produced buds shows a marked decline after the first week, reaching a minimum by the end of the second week of about one tentacle less than present on buds produced on Day 1. The analysis of variance shows that this change is highly significant ($0.001 > P$) and largely attributable to linear regression, also highly significant ($0.001 > P$). These data were corrected for differences in the sizes of the daily samples (sub-classes). The reduction in the sum of squares due to fitting constants was calculated and the interaction sum of squares obtained by difference from the sum of squares for subclasses (Snedecor, 1956). The results of these calculations had little impact on the analysis, and the interaction term was not significant.

Columns 4 and 5 (Table V) list the number of buds produced by the 12 animals on each feeding schedule on each day of the two week period. The total number of buds produced by animals on each schedule did not differ significantly ($0.10 > P > 0.05$), but the number of buds produced by three of the pairs differed (indicated by asterisks) at the 0.05 confidence level or below when tested by Chi-square. The null hypothesis, that the differences between these paired data are no greater than that expected as a result of random error, is, therefore, rejected in these cases. Feeding 3 days in succession (MTW) seems to have cycled budding so that the detachment of buds peaked on weekends and diminished mid-week. The different rates of budding on animals in the two schedules contrasts with the similarity in the tentacle numbers on the buds, and their decline in tentacle number over time. The tentacle number on freshly produced buds undergoing a reduction in feeding schedule from daily to 3-days a week, therefore, is influenced by caloric intake per week, but not by how the dose of food is supplied.

In another experiment animals were fed twice a day for a comparison with controls fed only once a day. Allowing the animals one week to acclimatize, data on bud

TABLE V

Average tentacle number on 24 parents and their buds, and number buds produced whole on two feeding schedules.

Day	Tentacle number on parents	Tentacle number on buds	Feeding schedules	
			MTW number buds	MWF number buds
0 Su	6.50			
1 M	6.50	6.48	11	12
2 Tu	6.46	6.50	6	8
3 W	6.46	6.57	7	7
4 Th	6.38	6.35	12	8
5 F	6.42	6.57	11	3*
6 Sa	6.29	6.10	12	9
7 Su	6.33	5.83	13	5*
8 M	6.46	5.75	6	6
9 Tu	6.46	5.80	1	4
10 W	6.46	5.86	4	10
11 Th	6.46	6.41	9	8
12 F	6.46	5.25	7	1*
13 Sa	6.46	5.33	9	3
		Total	108	84

* Difference between paired numbers of buds significant as tested by Chi-square.

production and tentacle number on the seventh and eighth days after commencing the twice-daily feeding schedule were accumulated for analysis. In contrast to the 12 controls which formed 22 buds, the animals fed twice-daily formed 45 buds, twice as many and a highly significant difference. The control population's tentacle number on buds produced was 6.04 and stable around this mean, but the animals fed twice-daily had a mean tentacle number on buds produced of only 5.36, a highly significant difference. Animals fed twice-daily form buds that begin budding themselves sooner, indeed, even while still attached to the parental animals, compared to animals fed once-daily. Thus, feeding animals twice daily to repletion increases size, rate of budding, shortens the time of onset of budding, but decreases tentacle number on buds compared to animals fed only once-daily. The tentacle number on the parental animals of both sample populations did not change significantly over the course of the week, despite the conspicuous change in the size of the animals fed twice-daily.

Effects of changing temperature, wounding and grafting

In addition to excess feeding, the average size of hydras is increased by decreasing temperature (Stiven, 1965; Park and Ortmeyer, 1972; Bisbee, 1973). One of us (D.M.) raised hydras at three temperatures, 16, 21, and 26° C, and determined the tentacle numbers for 12 parents and the buds produced over 8 days at each temperature. In no case did the parent's tentacle number change, but the tentacle numbers of freshly produced buds changed whenever the temperature had changed from the initial condition. The average tentacle number on freshly produced buds on the seventh and eighth days of incubation at the respective temperatures were

TABLE VI

Average tentacle number before and after regeneration.

Number animals	Originally	After regenerations
8	5	6.000*
25	6	5.440*
16	7	5.875*

* Probability that difference resulted from random error less than 0.01.

6.67, 6.33, and 6.04 tentacles per bud. The analysis of variance failed to indicate significant differences among these means, but linear regression was significant and accounted for almost all the variation in tentacle numbers between the means. The regression equation is $Y = 7.65 - 0.062X$, where Y is tentacle number and X is temperature in degrees centigrade. The Q_{10}, or temperature coefficient, is well in the range of expectations for enzymatically mediated biological reactions (Mitchell, 1950), but because fewer tentacles are produced at higher temperatures, suggests inhibition.

Cutting animals also reduces their overall size and ordinarily results in regeneration. When proximal pieces are preserved, tentacles regenerate at their distal wound surfaces. In order to compare the frequencies of tentacle numbers regenerated on proximal pieces of animals cut to different sizes, "standard" animals were cut through their gastric regions (the region between the head and the budding region), either directly below the head (headless animals) or mid-way down the gastric region. The proximal pieces of headless animals are approximately half again as large as the proximal pieces of animals cut mid-gastrically. Regenerated tentacles were counted daily until no further change in tentacle number was recorded (about four days). In a total sample of 82 animals no significant differences appeared in the mean tentacle numbers regenerated by animals cut at either level. The average tentacle number was 6.48 tentacles, despite the differences in size.

This result would seem to differ from that of Schaller (1973) who reports mean differences in tentacle numbers on animals wounded at different levels. However, since she employed nonbudding animals and cut them mid-way down the length of their body columns, she must have wounded them in a considerably lower region than we did here. Furthermore, she does not report that the tentacle numbers achieved were stable. It is well known that head structures in general regenerate at a slower rate at more proximal levels, especially in the peduncle, than at more distal levels of the gastric region (Webster, 1966). Finally, her report is ambiguous as to whether all her animals are starved one or two days prior to wounding, a consideration that could conceivably have significant effects on the frequency of tentacles regenerated inasmuch as feeding schedule effects tentacle number on buds.

In order to determine what relationship might exist between the number of tentacles originally present on an animal and the number regenerated, forty nine "standard" animals, chosen at random, had their tentacle number ascertained and then had their heads amputated. The mean number of tentacles originally present and those present after regeneration was complete are shown in Table VI. The asterisks indicate differences which are significant at the 0.01 level of confidence or below. An interesting feature of this result is that the mean tentacle numbers for all the regenerates do not differ significantly. This suggests that the regeneration of tentacles neither increases nor decreases tentacle number, but tends to regenerate tentacles regressively toward the mean for the population. Animals having tentacle numbers above the mean tend to regenerate fewer tentacles. In this case, the mean tentacle number of the regenerates was 5.57 tentacles. Since the mean tentacle number for the original sample was 6.16, significantly above the mean for regenerates, regeneration does not necessarily restore the mean originally present.

In order to create larger-than-normal animals additional gastric regions were grafted to headless animals thus lengthening the animals approximately a third again as much as they were originally. The distal head, present on the grafted gastric region, was then amputated and the regeneration of tentacles at this wound site monitored. Of 20 animals having an average original tentacle number of 6.45 tentacles, heads regenerated with an average tentacle number of 4.30, a highly significant decrease. In addition, five animals lengthened by grafting failed to regenerate heads at all even after a week. A total of six animals among the 25 regenerated so-called secondary heads (Shostak, 1972) at the border between the grafted pieces. The mean tentacle number for these secondary heads was 4.17 tentacles, not significantly different from the mean tentacle number regenerating on heads at the distal ends of the animals. Animals elongated by grafting, therefore, regenerate significantly fewer tentacles than normal at both distal wound sites and at graft borders.

Headless animals may also be grafted together in a "head-to-head" fashion, thus making an animal about twice the normal length, but with feet at both ends. Such animals regenerate heads at the border between the graft pieces, and in about half of the animals form a single integrated head rather than two heads (for a similar case see Shostak, 1977a). For 18 such grafts regenerating single heads, a mean tentacle number of 8.50 was found, a highly significant increase compared to the mean of 6.11 for the original heads, prior to amputation, but, certainly nowhere near a doubling of the original tentacle number. Among these, animals with 9 to 11 tentacles were selected (a sample of 7) and had their new heads amputated. Beginning with a mean tentacle number of 9.57, these animals regenerated tentacles with a mean tentacle number of 8.29, a significant diminution. Here again, tentacle numbers of regenerates tend to regress toward a normal value, but evidently, the diminution of tentacle numbers only proceeds at the rate of one to two tentacles per regeneration.

Discussion

Hydra has long figured in the pursuit of morphogenically significant substances, but has recently received wide spread attention due to the success of studies apparently demonstrating the morphogenic activity of neurosecretory substance. Schaller (1973; 1976a, b) has reported fractional increases in the mean tentacle numbers of samples of regenerating animals exposed to what are reported to be moderately purified neurosecretory products, compared to samples of other untreated regenerating animals. In order to evaluate such reports, one would like to know how tentacle number is distributed in the normal population and how its mean and variation are determined.

The present study agrees with two earlier studies (Parke, 1900; Liu and Chang, 1946) that tentacle number varies widely in populations of *Hydra*. Parke, who studied *H. fusca* and *H. viridis* from the wild, however, seems to have had difficulty culturing these animals and acknowledges the occurrence of greatly distorted animals whose regulation of form is discussed at length. Liu and Chang studied *H. vulgaris* and, as in the present study of *H. viridis*, found tentacle number normally distributed in their population. Since the introduction of adequate methods for mass-culturing *Hydra* in the laboratory by Loomis and Lenhoff (1956) the appearance of abnormal animals is rare, especially in rapidly budding colonies not heavily infested with endemic parasites. The variation of tentacle number found in the present study, therefore, is not attributed to poor conditions or variations found among wild populations. Even under vigorously controlled laboratory conditions, the tentacle number of *H. viridis* is normally distributed.

The present report also confirms Liu and Chang's (1946) conclusion that tentacle number is fixed prior to bud detachment, indeed, as much as a day prior to detachment. Following this time, an animal's tentacle number may be considered a stable characteristic, barring amputation or the extreme conditions known as depression, which presumably affected Parke's animals. Animals in the present study resisted changes in tentacle number for periods of observation as long as two weeks during which feeding was reduced or temperature was altered. Tentacle number seems to be fixed with a degree of determination which suggests structural rigidity. Variation in tentacle number found in the populations produced under adequate circumstances cannot be attributed to changes in individuals' tentacle numbers, but must be due to variation resulting from the production of buds with different tentacle numbers.

The freshly detached buds collected in the present study had tentacle numbers which were normally distributed. Evidently, the development of tentacles on buds is subject to controls that concentrate animals at the mean tentacle number and provide fewer animals with other tentacle numbers. One might expect that the control of the mean tentacle number is genetic and inherited and that variation is due to random error around a mean. The present results on the effect of size, temperature, feeding schedule, and wounding and grafting indicate that setting the mean tentacle number is more complex.

A variety of conditions were found to alter the mean tentacle number of freshly produced buds. We were especially interested in conditions which were known to alter the size of the budding animal, since Parke (1900) had reported that tentacle number varied with size and changed as a function of size. Otto, Dunne, Wirth, and Campbell (1976) and Otto and Campbell (1977) showed that the size of hydras is correlated with the size of buds, albeit they failed to report on tentacle numbers. The present study, likewise, shows that larger animals, cultured at 16° C, produced buds with more tentacles than smaller animals, cultured at 26° C. Such results are clearly suggestive of so-called mass phenomena in which the density, mass, or volume of cells present at a time has its own morphogenic consequences. The negative relationship between temperature and tentacle number suggests that metabolic rate is inversely proportional to tentacle development, or that products of metabolism are inhibitory to tentacle formation. The rate at which animals grow, determined by the frequency at which they are fed, controls the rate of budding and may have indirect consequences for tentacle development. Animals fed more frequently than once a day bud so rapidly that buds begin budding before actually detaching from their parents. Growth that is overwhelmingly directed to budding may divert cells into buds that are otherwise destined to take part in tentacle formation. When animals are fed less frequently than once a day, fewer cells may be available for recruitment into tentacles. The genetic constitution of parental animals is difficult to contemplate in the absence of specific information, but since buds are produced vegetatively one would expect that the buds and parents would have the same genetic complexion. Our finding that the tentacle number on buds is negatively correlated with the tentacle number of parents in the sample of animals tested suggests just how complex the problem of genetic control of tentacle number may be. Finally, as was first pointed out by Liu and Chang (1946), a biological parameter which is normally distributed, with a mean and a standard deviation, is inevitably genetically determined although the mechanism for fixing tentacle number in any animal may be subject to the effects of polygenes and modifier genes as well as to environmental conditions. The setting of a population's mean tentacle number, therefore, would seem to represent the culmination of the interactions of a variety of factors.

Thresholds would presumably be a part of any endogenous control system generating buds with different numbers of tentacles. Such a control system has already been hypothesized to operate in determining the production of a hydra's foot (MacWilliams, Kafatos, and Bossert, 1970). There, an inhibitor of foot formation is thought to be released by the foot with continuously variable intensity controlling foot regeneration and normal maintenance. Given this perspective, one can imagine an inhibitor of tentacle number whose quantitative production is sensitive to physiological and environmental cues, but which operates a mechanism inhibitable at given threshold levels of concentration. One level might determine that six tentacles are produced, another level that five tentacles are produced and so on. The neurosecretory substances investigated by Schaller (1976a, b) are not thought to play inhibitory roles, however. Such a role may be played by another substance in her scheme. Rather, neurosecretory substances are thought to promote or determine tentacle development. Alternatively, physiological and environmental cues might alter thresholds rather than levels of inhibitors or determinants, or both thresholds and levels of substances responsible for controlling tentacle number. This possibility would seem to have sufficient modality to encompass the present results.

A complex system for determining tentacle number would seem well suited for hydra's way of life, which subjects it to wide and repeated variations in environmental conditions. Presumably, tentacle number is a crucial adaptation for hydras in the wild and is, therefore, subject to natural selection and hereditary control. Once fixed, a bud's tentacle number remains constant except under conditions initiating regeneration. An optimal tentacle number in one environment may not be optimal in another, however, and a system for changing the mean tentacle number would also be adaptive. We have seen that the animals generate buds with different tentacle numbers which, by chance might have selective advantages in terms of leaving progeny in the different environments they encounter. Natural selection might then shape the population preferentially according to local conditions, allowing animals with the optimal tentacle number for these conditions to survive while eliminating animals with nonoptimal numbers. Parke (1900), not only observed that the tentacle numbers of *H. fusca* differed from those of *H. viridis,* but different populations of members of the same species taken from different locales also had different mean tentacle numbers. But, *Hydra* is not totally dependent on random variation for generating animals with different tentacle numbers. The mean tentacle number on buds produced under different conditions is capable of shifting in predictable ways. Replacement populations produced by budding may have mean tentacle numbers more nearly at the optimal level for particular local conditions, and contain fewer individuals with nonoptimal tentacle numbers than would be produced merely by a random system for generating variation.

Regeneration of tentacles also seems adaptive for altering the mean tentacle number of a population. The number of tentacles regenerating on distal pieces tends to gravitate toward a mean, characteristic of the population, not necessarily resembling that of the original population, and not conspicuously sensitive to size. The number of tentacles regenerating on wounded gastric regions can actually exceed that number originally present when that number was above the mean. Even animals with abnormally high numbers of tentacles (produced by the production of a single head from two regenerating surfaces brought together by grafting) have fewer tentacles than originally present on the two heads separately, and regenerate tentacles with more nearly normal tentacle numbers than they had prior to amputation. *Hydra,* thus, seems to "know" how many tentacles to produce in a given environment and under particular physiological conditions, and regenerates tentacles in increasingly close approximations to that number as opportunities made available by budding and amputation permit.

Regeneration is conspicuously similar to budding as a nexus for altering tentacle number. Furthermore, like the hydras enlarged by feeding twice a day which produce buds with fewer tentacles than animals fed once a day, hydras enlarged by grafting regenerate fewer tentacles than pieces of normal animals. But, unlike budding, the role of regeneration in the wild is unknown. Except for adverse conditions causing depression and subsequent regulation, regeneration as such may be infrequent in wild populations. Budding, rather than regeneration would seem to be the general route for regulating tentacle number in wild populations.

The present study should have some impact on those studying morphogens, whether simple or interacting, inhibitors or stimulators, especially those employing regenerating hydras as their test system. If hydras are "imperfect test tubes" (Schaller, 1976a, p. 1), it is because *Hydra* has evolved mechanisms for moderating the effects of environmental contingencies. *Hydra* may meet challenges, both in the wild and in the laboratory, in ways contrary to expectations. Relying on its own wisdom, *Hydra* has evolved systems for rigidly maintaining the tentacle number of individuals despite moderate changes in environmental conditions, for generating buds with a normal distribution of tentacle numbers, and for regenerating heads with tentacle numbers that may be different from those originally present. Among the things a hydra "knows" is how to produce buds with different mean numbers of tentacles and to regenerate tentacles in numbers that increasingly approximate a mean dictated in part by particular conditions. One might be rewarded by efforts to understand a hydra's wisdom in stabilizing tentacle numbers on individuals while providing a mechanism for generating variety and shifting means.

The present report makes clear, however, that relying on small fractional differences in tentacle numbers to draw large inferences about the operation of morphogenically significant substances in *Hydra* is not prudent.

Summary

1. The distribution of tentacle numbers on *Hydra viridis,* cultured in the laboratory were studied in order to gather some appreciation of the normal range of tentacle number, and how that number is influenced by vegetative reproduction, and by contingencies which alter the size of a hydra.

2. Large variation among individuals selected at random was found to be the predominant statistical factor in the analysis of variance of tentacle number and cautions against drawing inferences from small differences in sample mean tentacle numbers. Tentacle number is normally distributed and stable for each individual from the day before detaching as a bud for periods as long as 2 weeks despite changes in feeding schedule and temperature.

3. The mean tentacle number on samples of buds produced over time is subject to change. Tentacle number on buds can be negatively correlated with tentacle number on parents and decreases both when the normal daily feeding schedule is changed to a 3-days a week schedule (whether in succession or with intervals between feedings), or increased to twice-daily feeding. Buds produced at higher temperatures also have lower tentacle numbers. Conditions which might raise the metabolic rate of the parent hydras, therefore, can reduce tentacle numbers on buds.

4. Hydras wounded through the gastric region regenerate the same number of tentacles despite differences in size. Regeneration yields tentacle numbers tending to gravitate toward a mean for the population independent of the original tentacle number. Both increases and decreases are recorded. Animals enlarged by grafting, however, tend to regenerate fewer tentacles than originally present even when double-heads regenerate as one. Regeneration, like budding, can alter the population's mean tentacle number according to prevailing conditions.

LITERATURE CITED

Bisbee, J. W., 1973. Size determination in *Hydra*: the roles of growth and budding. *J. Embryol. Exp. Morphol.,* **30**: 1–19.

Burnett, A. L., 1961. The growth process in Hydra. *J. Exp. Zool.,* **146**: 21–84.

Burnett, A. L., 1967. Control of polarity and cell differentiation through autoinhibition—a model. *Exp. Biol. Med.,* **1**: 125–140.

Campbell, R. D., 1967. Tissue dynamics of steady state growth in *Hydra littoralis.* II. Patterns of tissue movement. *J. Morphol.,* **121**: 19–28.

Corff, S. C., and A. L. Burnett, 1969. Morphogenesis in *Hydra.* I. Peduncle and basal disc formation at the distal end of regenerating hydra after exposure to colchicine. *J. Embryol. Exp. Morphol.,* **21**: 417–443.

Lenicque, P. M., 1967a. Action of thalidomide on the induction of tentacles in regenerating *Hydra littoralis. Acta Zool.,* **48**: 127–139.

Lenicque, P. M., 1967b. Note sur l'utilisation des hydroides pour l'etude et l'action des medicaments sur le developpement organique. *Therapie,* **22**: 411–418.

Lenicque, P. M., and M. Lundblad, 1966. Promotors and inhibitors of development during regeneration of the hypostome and tentacles of *Hydra littoralis. Acta Zool.,* **47**: 277–287.

Lentz, T. L., and R. Barrnett, 1963. The role of the nervous system in regenerating hydra: the effect of neuropharmacological agents. *J. Exp. Zool.,* **154**: 305–327.

LESH, G. E., AND A. L. BURNETT, 1964. Some biological and biochemi al properties of the polarizing factor in hydra. *Nature*, **204**: 492.

LESH-LAURIE, G. E., 1974. Tentacle morphogenesis in hydra: a morphological and biochemical analysis of the effect of actinomycin D. *Am. Zool.*, **14**: 591–602.

LIU, T. T., AND J. T. CHANG, 1946. Number of tentacles in *Hydra vulgaris* as a genetic character. *Nature*, **157**: 728.

LOOMIS, W. F., AND H. M. LENHOFF, 1956. Growth and sexual differentiation of hydra in mass culture. *J. Exp. Zool.*, **132**: 555–573.

MACWILLIAMS, H. K., F. C. KAFATOS, AND W. H. BOSSERT, 1970. The feedback inhibition of basal disk regeneration in *Hydra* has a continuously variable intensity. *Dev. Biol.*, **23**: 380–398.

MITCHELL, P. H., 1950. *A textbook of biochemistry.* McGraw Hill Book Company, Inc., New York, 695 pp.

MÜLLER, W. A., AND K.-D. SPINDLER, 1971. The "polarizing inducer" in hydra: a re-examination of its properties and its origin. *Wilhelm Roux's Arch. Entwicklungsmech. Org.*, **167**: 325–335.

OTTO, J. J., AND R. D. CAMPBELL, 1977. Budding in *Hydra attenuata*: bud stages and fate map. *J. Exp. Zool.*, **200**: 417–428.

OTTO, J. J., J. DUNNE, P. WIRTH, AND R. D. CAMPBELL, 1976. Tissue behavior during budding in *Hydra attenuata*. *Am. Zool.*, **16**: 186.

PARK, H. D., AND A. B. ORTMEYER, 1972. Growth and differentiation in hydra: II. The effect of temperature on budding in *Hydra littoralis*. *J. Exp. Zool.*, **179**: 283–288.

PARKE, H. H., 1900. Variation and regulation of abnormalities in hydra. *Wilhelm Roux' Arch. Entwicklungsmech. Org.*, **10**: 692–710.

SCHALLER, H. C., 1973. Isolation and characterization of a low-molecular weight substance activating head and bud formation in hydra. *J. Embryol. Exp. Morphol.*, **29**: 27–38.

SCHALLER, H. C., 1976a. Action of the head activator as a growth hormone in hydra. *Cell Differ.*, **5**: 1–11.

SCHALLER, H. C., 1976b. Head regeneration in hydra is initiated by the release of head activator and inhibitor. *Wilhelm Roux's Arch. Dev. Biol.*, **180**: 287–295.

SHOSTAK, S., 1968. Growth in *Hydra viridis*. *J. Exp. Zool.* **169**: 431–446.

SHOSTAK, S., 1972. Inhibitory gradients of head and foot regeneration in *Hydra viridis*. *Dev. Biol.*, **28**: 620–635.

SHOSTAK, S., 1977a. Regeneration in *Hydra viridis* with multiple-gastric region grafts: reversal of the middle piece. *J. Morphol.*, **154**: 1–18.

SHOSTAK, S., 1977b. Vegetative reproduction by budding in *Hydra*: a perspective on tumors. *Perspect. Biol. Med.*, **20**: 545–568.

SHOSTAK, S., AND R. V. TAMMARIELLO, 1969. Supernumerary heads in *Hydra viridis*. *Natl. Cancer Inst. Monogr. (U.S.A.)*, **31**: 739–750.

SHOSTAK, S., N. G. PATEL, AND A. L. BURNETT, 1965. The role of mesoglea in mass cell movement in *Hydra*. *Dev. Biol.*, **12**: 434–450.

SHOSTAK, S., J. W. BISBEE, C. ASHKIN, AND R. V. TAMMARIELLO, 1968. Budding in *Hydra viridis*. *J. Exp. Zool.*, **169**: 423–430.

SNEDECOR, G. W., 1956. *Statistical methods.* The Iowa State University Press, Ames, Iowa, 534 pp.

SOKAL, R. R., AND F. J. ROHLF, 1969. *Biometry.* W. H. Freeman and Company, San Francisco, 776 pp.

STIVEN, A. E., 1965. The relationship between size, budding rate, and growth efficiency in three species of hydra. *Res. Popul. Ecol. (Kyoto)*, **7**: 1–15.

TURING, A., 1952. The chemical basis of morphogenesis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.*, **237**: 37–72.

WEBSTER, G., 1966. Studies on pattern regulation in hydra. III. Dynamic aspects of factors controlling hypostome formation. *J. Emb. Exp. Morphol.*, **16**: 123–141.

WEBSTER, G., 1967. Studies on pattern regulation in hydra. IV. The effect of colcemide and puromycin on polarity and regulation. *J. Emb. Exp. Morphol.*, **18**: 181–197.

YASUGI, S., 1974. Observations on supernumerary head formation induced by lithium chloride treatment in the regenerating hydra, *Pelmatohydra robusta*. *Dev. Growth Differ.*, **16**: 171–180.